Journal of
Fisheries and Aquatic Science

ISSN 1816-4927

Academic
Journals Inc.

www.academicjournals.com

Journal of Fisheries and Aquatic Science 9 (1): 33-39, 2014
ISSN 1816-4927 / DOI: 10.3923/jfas.2014.33.39

Specific Primers of *Oryzias javanicus*, *O. dancena*, *O. minutillus* and *O. woworae* and Molecular Phylogeny Using Partial Mitochondrial DNA Sequences

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ABSTRACT

Fish of the genus *Oryzias* (Teleost) are one of the experimental animals used in various studies of biological vertebrates. These fish mostly inhabit the fresh and brackish waters of East and Southeast Asia. In the present study, specific primer pairs were used to identify the four species of *Oryzias* (*O. javanicus*, *O. dancena*, *O. minutillus* and *O. woworae*) using PCR (polymerase chain reaction) analysis. Moreover, the phylogenetic relationship of *O. woworae* within the eleven *Oryzias* species was assessed based on the complete nucleotide sequence of tDNA-Val and the partial sequences 12S ribosomal DNA (rDNA) and 16S rDNA. The PCR results showed individual amplification by the specific primers in the four species. The phylogenetic data, which clustered into three groups, confirmed the hypothesis that the *Oryzias* taxa referred to those sequences. These data give a set of molecular tools and a source of nucleotide data for *Oryzias* that might increase the knowledge distribution of molecular genetics in the Adrianichthyidae.

Key words: Specific DNA markers, *Oryzias*, molecular phylogeny, partial mitochondrial DNA

INTRODUCTION

Oryzias spp. (also called ricefish or medaka in Japanese) belong to the family Adrianichthyidae and are a model organism used in the fields of embryology, genetics and toxicology (Matsuda *et al*., 2002; Zhang *et al*., 2008; Ngamniyom *et al*., 2009). These fish are broadly distributed throughout East, South and Southeast Asia and the Indo-Malay-Philippines Archipelago. There are twenty-two recognised species in this genus (Parenti, 2008). Takehana *et al*. (2005) reported that the zoogeographical distribution is associated with the phylogenetic relationship of the *Oryzias* taxa inferred from mitochondrial DNA.

In Southeast Asia, *O. javanicus* (Java-medaka) and *O. dancena* predominantly inhabit the brackish waters South of Thailand and Malaysia, exhibiting seawater adaptability and serving as a fish model for osmoregulation (Inoue and Takei, 2002; Yang *et al*., 2013). *O. minutillus* (dwarf medaka or Thai medaka) is the smallest fish in the genus *Oryzias* and widely distributed in Thailand (Ngamniyom and Panyarachun, 2012a). This species is a sensitive bioindicator of xenoestrogenic activity in the natural environment and laboratory (Ngamniyom *et al*., 2011; Ngamniyom and Panyarachun, 2012b). In Indonesia, *O. woworae* (Daisy's ricefish), which is

remarkably colourful, is a native fish in a freshwater habitat on the main island of Sulawesi (Parenti and Hadiaty, 2010). This species might serve as an alternative Teleost to use as experimental model with many advantageous characters.

DNA genetic markers are an important tool for fish identification, especially in an aqua-cultural population and community (Liu and Cordes, 2004). Termvidchakorn and Magtoon (2008) described the criteria of species identification in the developmental stages of *Oryzias* larvae from Thailand. However, accurate identification using molecular genetics still needs to bedeveloped.

In this study, specific primers were chosen based on partial mitochondria DNA sequences (the complete tDNA-Val (transfer RNA-valine) sequence and partial 12S ribosomal DNA (rDNA) and 16S rDNA sequences) for the identified *O. javanicus*, *O. dancena*, *O. minutillus* and *O. woworae*. Furthermore, the phylogenetic relationship of *O. woworae* within the *Oryzias* species was evaluated using these nucleotide sequences.

MATERIALS AND METHODS

Fish collection: *O. javanicus* and *O. dancena* (Fig. 1a, b) were collected from the brackish waters of mangrove from the Satun Province, South of Thailand (6°37'26"N 100°04'01"E). *O. minutillus* (Fig. 1c) were collected from the fresh water of the Nakhon Nayok Province, Centre of Thailand (14°12'44"N 101°12'06"E). *O. woworae* were purchased from a commercial source in Bangkok, Thailand (Fig. 1d). These fish were immediately preserved in 100% ethanol and stored at -20°C.

Genomic DNA extraction and PCR analysis: The genomic DNA of each specimen was extracted using a DNeasy Tissue kit (Qiagen, Germany) according to the manufacturer's protocol. In *O. woworae*, mitochondrial DNA was amplified by Taq DNA polymerase (Takara, Tokyo, Japan) and contained the complete tDNA-Val sequence and partial 12S rDNA and 16S rDNA sequences. The primer pairs used in this study are shown in Table 1. The PCR conditions were the following: An initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 1 min. A final extension was performed at 72°C for 10 min. The PCR products were electrophoresed on a 1.5% agarose gel that was ethidium bromide stained and visualised under a UV transilluminator. The amplified PCR products were

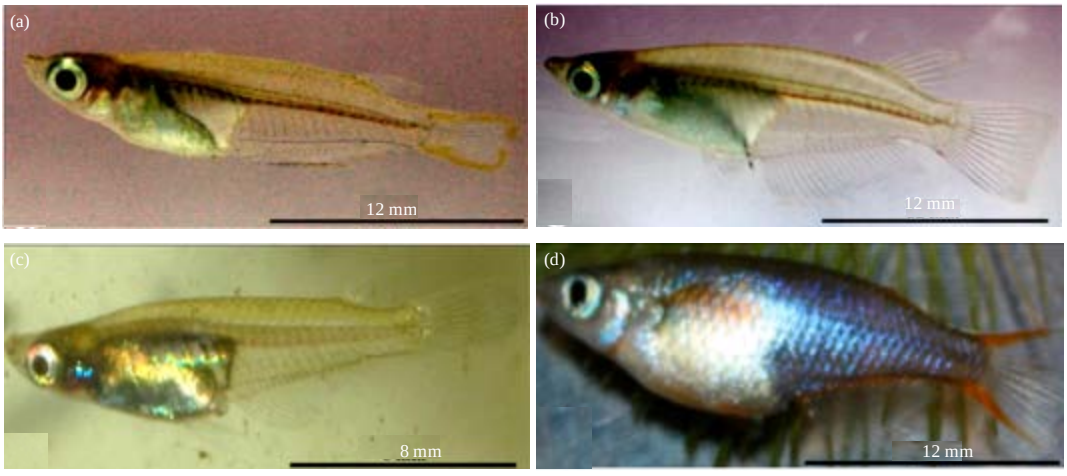

Fig. 1(a-d): Four fresh samples of Southeast Asian *Oryzias*: (a) *O. javanicus*, (b) *O. dancena*, (c) *O. minutillus* and (d) *O. woworae*

Table 1: Primer pairs used in PCR and sequencing reaction of four *Oryzias*

Species	Primer names	Primers	For using in	Product (bp.)
O. javanicus	Ojsfforward	(fwd): 5'-GCCCCTTCTATCAAAAGCCGC-3'	Specific primer	1371
	Jsrreward	(rev):5'-GGATTGCGCTGTTATCCCTGG-3'		
O. dancena	Odsf	fwd: 5'-CCAGCCGAATGTAACCGCTAAC-3'	Specific primer	600
	Jsr	rev:5'-GGATTGCGCTGTTATCCCTGG-3'		
O. minutillus	Omsf	fwd:5'-AGCACAAGTGTAAGTCGGAGTGAAC-3'	Specific primer	664
	Omsr	rev:5'-CCCCCTTTTTATGGACTGTCTGAGG-3'		
O. woworae	Oj1	fwd:5'-AGAGAGTCCTGCTGAAACTGGCCCTA-3'	Sequencing	1729
	Oj2	rev: 5'-AGAAACTGACCTGGATTACTCCGGTC-3'		
	Oj3	ffwd:5'-GCACTATAGTCTGGACCCCCG-3'	Sequencing	746
	Oj4	rrev: 5'-CGGCCGTTAAACGTTAGAGTC-3'		
	Owsf	fwd:5'-GCACAACTGAGTTGTTAGACACC-3'	Specific primer	958
	Jsr	rev:5'-GGATTGCGCTGTTATCCCTGG-3'		

Table 2: GenBank accession number of fish species for phylogenetic

Analysis

Species	Accession No.	References
Ingroup		
Oryzias sarasinorum	AB370891	Setiamarga *et al.* (2008)
O. celebensis	AB498070S	Etiamarga *et al.* (2009)
O. marmoratus	AP005981	Setiamarga *et al.* (2009)
O. latipes	AP008942	Hirayama *et al.* (2010)
O. latipes shanghai	AP008948	Hirayama *et al.* (2010)
O. sinensis	GU013788	Yoon *et al.* (2011)
O. luzonensis	AB498064	Setiamarga *et al.* (2009)
O. dancena	AB498069	Setiamarga *et al.* (2009)
O. javanicus	AB498067	Setiamarga *et al.* (2009)
O. minutillus	AB498068S	Etiamarga *et al.* (2009)
O. woworae	KC517074	In this study
Outgruop		
Cypselurus hiraii	AB182653	Nagase *et al.* (2005)
Gambusia affinis	AP004422	Miya *et al.* (2003)

purified using the QIAquick Gel Extraction Kit (Qiagen, Germany). Nucleotide sequencing was performed by the Macrogen DNA Sequencing Service, Korea. The partial mitochondrial DNA sequence of *O. woworae* has been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) (accession number KC517074).

Phylogenetic analysis: The DNA sequences of the four *Oryzias* species (accession number in Table 2) were aligned using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to identify regions of local differences for designing specific primers. The PCR cycle conditions for the specific marker test followed the above conditions. For a phylogenetic evaluation within the *Oryzias* species (Table 2), an analysis of the neighbour-joining with Kimura's 2-parameter correction was carried out using the Molecular Evolutionary Genetics Analysis (MEGA) version 5.10 software package (Tamura *et al.*, 2011). Finally, the sequences from each amplified band were confirmed using the online program Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

DNA specific marker test: In *O. javanicus*, the amplification band of the PCR product was thick and identified by the specific markers from Ojsf and Jsr (Fig. 2a). There were no amplification bands in *O. dancena*, *O. minutillus* and *O. woworae*. The primer pairs from Odsf and Jsr amplified the sequences of *O. dancena* but did not amplify those of *O. javanicus*, *O. minutillus* and *O. woworae* (Fig. 2b). The genomic DNA of *O. minutillus* was amplified using primers from Omsf and Omsr (Fig. 2c). In contrast, amplified bands were absent in *O. javanicus*, *O. dancena* and *O. woworae* using these primers. In *O. woworae*, an individual band of a DNA fragment was shown following amplification by the Owsf and Jsr primers, whereas no evidence of DNA amplification was observed in *O. javanicus*, *O. dancena* and *O. minutillus* (Fig. 2d).

Phylogenetic tree: The phylogenetic relationship of eleven *Oryzias* and two outgroup species was analysed based on the complete tDNA-Val sequence and partial 12S rDNA and 16S rDNA sequences. The results showed a common pattern of cladistics in the *Oryzias* species and the clades

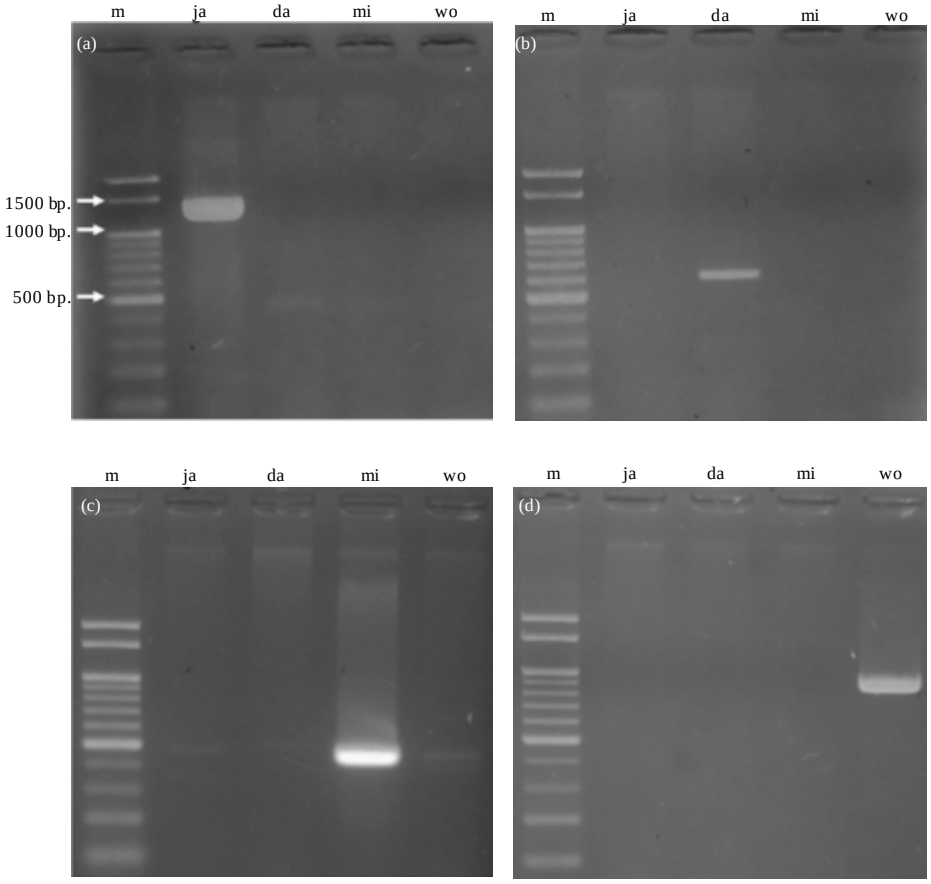

Fig. 2(a-d): PCR analysis with results gel electrophoresis using the specific primers. The primer of Ojsf and Jsr for *O. javanicus* (ja) (a), Odsf and Jsr for *O. dancena* (da) (b), Omsf and Omsr for *O. minutillus* (c), Owsf and Jsr for *O. woworae* and (d) 1 kb of molecular marker (m)

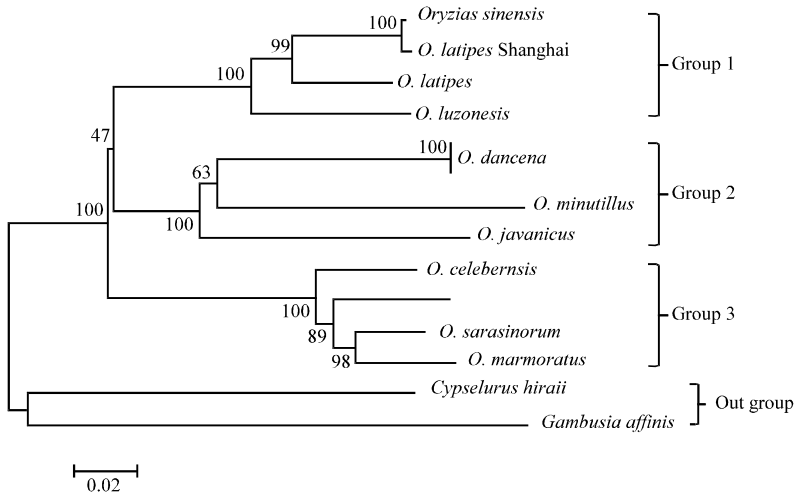

Fig. 3: Neighbour-joining tree with bootstrap test of 1000 replicates among the relationship of 11 *Oryzias* fish. Number on the branch tree was percentage of bootstrap. Bar indicates a corresponding of a length to 0.02 nucleotide substitution pers site

were far from the outgroups, *Cypselurus hiraii* and *Gambusia affinis* (Fig. 3). In this phylogeny, the *Oryzias* species were separated into three monophylogenetic groups. Group 1 consisted of *O. sinensis*, *O. latipes, O. latipes* (Shanghai stain) and *O. luzonesis*, group 2 consisted of *O. dancena*, *O. minutillus* and *O. javanicus* and group 3 consisted of *O. celebensis*, *O. woworae*, *O. sarasinorum* and *O. marmoratus*.

DISCUSSION

The primer pairs designed in this study were able to specifically amplify the partial mitochondrial sequences of two closely related species, *O. javanicus* and *O. dancena* (Yusof *et al*., 2012) and were not associated with cross-amplification in *O. minutillus* and *O. woworae*. The embryo, fry and juvenile are hard to distinguish by species; however, Termvidchakorn and Magtoon (2008) provided a method for identifying *Oryzias* larvae by developmental morphology. These results demonstrated a molecular biology tool that can be used for the precise identification of four *Oryzias* species. Genetic marker development has been applied in fisheries and aquaculture studies; examples of these markers include mitochondrial DNA, microsatellites, Single-nucleotide Polymorphisms (SNPs) and Random Amplification of Polymorphic DNA (RAPD) (Chauhan and Rajiv, 2010). This study reported one potential use of the specific primer sets: Identifying *O. javanicus*, *O. dancena*, *O. minutillus* and *O. woworae*.

Based on the partial mitochondrial sequence data, the phylogenetic analysis showed three clusters of *Oryzias*. *O. woworae* was monophyletic in the *O. celebensis* group taxa. This result corresponds to the previous study of Herder *et al*. (2012), which showed that *O. woworae* was part of the *Oryzias celebensis* group in a Bayesian phylogeny based on 16S rDNA sequences. In this study, *O. sinensis*, *O. latipes, O. latipes* Shanghai and *O. luzonesis* formed the Japanese medaka group. *O. dancena*, *O. minutillus* and *O. javanicus* formed the Java medaka group. This taxon data supported the study of Takehana *et al*. (2005), which hypothesised the relationships of fifteen medaka species in the *latipes*, *Celebensis* and *Javanicus* species groups using nuclear and mitochondrial sequences.

Based on the present knowledge, a set of molecular tools and a source of partial mitochondrial nucleotide sequences were provided. The specific primer set was used to precisely identify the four species of Southeast Asian *Oryzias*, although the accurate identification for other species of *Oryzias* remains to study.

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